Preparation of Probiotic *Shrikhand* using selected *Lactobacillus strains* and evaluating effects of different botanicals on its viability

A

Synopsis

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Introduction

India is the largest milk-producing country with milk production in 2017-2018 being 176.3 million tonnes with a capital availability of 375 gms/day (www.nddb.org/information/ stats milk pro-India). Milk is normal mammary secretion derived from milking of a healthy milch animal without either addition thereto or extraction there from and free from colostrum (FSSAI, 2011). It is the primary source of nutrition for young mammals before they can digest other types of food. Milk provides complete nutrition in a balanced proportion, providing protein (casein), carbohydrate (lactose), calcium, vitamin B complex (thiamine, riboflavin, niacin, vitamin B6, and folate), vitamin A, vitamin C, zinc, and magnesium (Davoodi et al., 2013). Full cream (6% fat and 9% SNF) also provides essential fatty acid, conjugated linoleic acid (CLA) present as a minor component of milk fat is an antioxidant (Jelen 2005). Milk utilization pattern shows fluid milk consumption of 45% followed by 35% converted to butter or ghee and 7% made into the paneer and other cheese, 4% is dried into milk powder, and the balanced used for other fermented products such as dahi, yogurt and, indigenous products.

Fermented milk products are the most popular milk-based products in the world because of their high nutritional and therapeutic value. The subtropical climate of India made it essential to devise India's modes of milk preservation. Consequently, various fermented milk products that suited the needs and tastes of its inhabitants developed. Over the years, fermented mil became and indispensable supplement to the staple food consumed every day. Shrikhand is one of the indigenous fermented milk products of India (Aneja et al., 2002).

Shrikhand is derived from the Sanskrit word "shtrikharini", meaning a curd preparation with added sugar, flavouring materials, fruits, and nuts. Shrikhand is a semisolid, sweetish sour, wholesome indigenous fermented milk product popular in the states of Gujarat, Maharashtra, Karnataka and some parts of North India. Shrikhand, obtained from dahi, contains most of the valuable constituents of milk such as protein, fat, minerals, fat-soluble vitamins, and an appreciable number of B-complex vitamins, particularly riboflavin and folic acid.

In modern Indian society, there is an increase in awareness among consumers towards health-promoting probiotic foods. Consequently, the demand for these foods is steadily increasing in the country. The probiotics are known to be capable of establishing a desirable ecology among microorganisms in the

gastrointestinal tract of consumers thus producing beneficial health effects. According to the FAO/WHO (2002), Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host".

At present, it is recognized that an optimum balance in the microbial population in the human digestive tract is associated with good nutrition and health. Increasing evidence indicates that consumption of probiotics can help to maintain a favourable microbial profile and result in several therapeutic benefits. In recent years, probiotic bacteria have increasingly been incorporated into food dietary adjuncts. It has been recommended that foods containing such bacteria should contain at least 10 live organism per gram of product at the time of consumption in order to produce therapeutic benefits. The consumption of probiotic products with the incorporation of health-promoting cultures like *Bifidobacterium* and *Lactobacillus* help to maintain good health, restoring vigour and combating intestinal and other disorders (Anon, 2005).

The fermented milk and probiotics used for fermentative purposes may contribute to a variety of health benefits. Keeping the socio-economic attributes and consumer preferences in view, the development of suitable dairy products for the incorporation of the wild strains of Probiotics is the need in the present world. Shrikhand being the most popular indigenous fermented product of India, attempts have been made to make it more attractive for consumer acceptance by value addition into shrikhand (Srivastava and Goyal, 2007).

The most important factor in fermented dairy products containing Probiotics is to ensure the viability of the probiotic. This is because the bacteria need to survive in the food during shelf life and gastrointestinal digestion i.e., acidic conditions of the stomach and degradation by hydrolytic enzyme and bile salts in the small intestine. Commercially shrikhand produced may contain single probiotic strain or probiotic combinations. Thus, the addition of probiotics increases nutritional and therapeutic properties. It is important that probiotic shrikhand must contain living probiotic strain in adequate concentration at the time of consumption.

However, the key problems associated with incorporating probiotic bacteria into milk during fermentation are slow growth in milk and low survival rate during storage. One of the strategies applied to improve the growth of probiotic bacteria is the addition of prebiotic substances with proper selection

of starter cultures. “Prebiotic” defined as selective non-digestible carbohydrate food sources, are being increasingly used in promoting the proliferation of *Bifidobacterium* and *Lactobacilli* in probiotic shrikhand and many other supplements with an active component such as plant sources, phenolic compounds, and anti-oxidative substances (chia seed, flax seed, wheat bran, fennel, Ashwagandha, Ginger, and Basil), will be tested.

The potential medicinal plant extracts used as a source for a natural compound that acts as a new anti-functional agent. Research groups have occupied new anti-functional agents in different fields according to the samples used for the expression of anti-microbial activities. The anti-microbial activity found in plant extracts for folk medicine, essential oils are isolated with compounds such as alkaloids, flavonoids, sesquiterpene lactones, triterpenes among many others. Some general combinations should be applied such as common parameters, plant material and culture suitable in using techniques employed, growth medium along with microorganisms tested & enumerated.

This work will aim to evaluate the viability of probiotic bacteria during fermentation and storage and evaluate several physiochemical properties, sensory and shelf-life study preference of different probiotic shrikhand each made with a combination of probiotic culture and variety of plant sources.

Significance of Plant Sources

Chia Seed (*Salvia hispanica*)

Chia seed also called Mexican Chia or Salvia Chia, a species of flowering plant in the mint family (lamiaceae), grown for its edible seeds. Chia seeds are a rich source of fibre, protein, omega-3fatty acid in form of alpha-linolenic acid (ALA), calcium, magnesium and phosphorus. It also increases the resistance of probiotic to gastric juice and bile acid. It is also help in prevention of many diseases like CVS, diabetes, obesity cancer and gives to strength our immune system.

Flax Seed (*Linum usitatissimum*)

Flax, also known as common flax or linseed, is a member of the genus *Linum* in the family Linaceae. It is a good source of phenolic compounds known as lignans, non-starch polysaccharides, and proteins of high quality (Rubilar etal.2010). Non-digestible polysaccharides is known to have a prebiotic effect and additionally, flaxseed soluble fibre, often called mucilage, is proved to enhance lactic acid bacteria survival and growth in probiotic products.

Wheat Bran (*Triticum aestivum*)

Wheat Bran contain oligosaccharide act as prebiotic (a non-digestible food ingredients/soluble dietary fibre) and increase beneficial bacteria (Probiotic) amount in a large bowel, thus improving gut health (topping, 2007). It is also preventing gastric cancer, treating Irritable Bowel Syndrome (IBS), reducing the risk of haemorrhoids and hiatal hernia, and type 2 diabetes.

Ashwagandha (*Withania somnifera*)

Ashwagandha is a shrub that flourishes in India. Ashwagandha has many beneficial elements, including flavonoids. Ashwagandha contain different natural antioxidants: superoxide dismutase, catalase and glutathione peroxidase which are responsible as health promoters. It has been used to boost the immune system, improve memory and to promote overall wellness. (Mehta, 2013)

Fennel (*Foeniculum vulgare*)

Fennel is a flowering plant species in the carrot family. Fennel is a rich source of fibre, potassium, folate, vitamin C and phytonutrient content. Fennel contains significant amounts of fibre. It decreases the risk of heart diseases as it helps reduce the total amount of cholesterol in the blood. Fennel's prebiotic properties that help feed probiotic bacteria; help restore balance in turn help improve gut health.

Ginger (*Zingiber officinale*)

Ginger is a flowering plant belonging to the Zingiber acai family. Ginger may help increase movement through the digestive tract, suggesting that it may relieve or prevent constipation. The other properties are including antioxidant, anti-inflammatory, blood circulation and anti-cramp, anti-ulcer, anticholinergic, analgesic, antimicrobial, anti-stress, anti-cancer, immunity booster, anti-diabetic, regulation of blood pressure and treatment of cardiovascular diseases.

Basil (*Ocimum basilicum L.*)

Basil is a culinary herb of the family Lamiaceae is one of the most common plants used for flavouring many dairy products that contain probiotic bacteria. It can provide vitamins, minerals, and a range of antioxidants.

This work will aim to evaluate the viability of probiotic bacteria during fermentation and storage and evaluate several physiochemical properties, sensory and shelf-life study preference of different probiotic shrikhand each made with a combination of probiotic culture and variety of plant sources.

Objectives of the Study

1. To develop probiotic shrikhand using selected probiotic bacteria like ***Lactobacillus acidophilus, Lactococcus lactis, Lactobacillus bulgaricus and Bifidobacterium.***
2. To assess the viability of probiotic bacteria in shrikhand using different botanical variants.
3. Standardization and Quality check of various samples by assessing the physiochemical properties and microbial quality as per FSSAI standards.
4. To evaluate antimicrobial activity of the probiotic against pathogens.

Review of Literature

VP Jadha et al., 2019 reported on developing probiotic shrikhand using safflower milk blended in milk added probiotic culture, mango pulp for flavour and storage stability.

Rekhadevi, Apoorva et al., 2018 reported on soy milk and fruit based shrikhand. Fruit pulp was added to soy milk to enhance vitamin A and vitamin C in shrikhand.

Amar Baksshori et al., 2018 enhanced the viability of probiotics used by phenolic compounds, prebiotic and antioxidant substances to provide functional properties and additional nutrients for bacteria growth in probiotic yogurt, ice-cream and cheese.

Parisa Shokryazdan, Chin Chin Seio et al., 2014 isolated, identified and characterized some lactic acid bacterial strains from human milk, infant faeces, and fermented grape and dates, as potential probiotic with antimicrobial activity against some human pathogenic bacteria.

Nidhi Ojha, Dr. Ramesh Chandra et al., 2018 reported on valuation by adding tulsi and turmeric powder into shrikhand honey used as sweeteners and analysed quality characteristics.

U.B. Landge, B.K. Pawar et al., 2011 assessed the suitability of adding ashwagandha powder 03%, 0.5%, and 0.7% with 40% can sugar (by weight of chakka) in shrikhand.

John David et al., 2018 incorporated aqueous moringa leaf powder @ 1%, 2% and 3% in shrikhand respectively and evaluated various parameters.

Material and Method

Collection of Plant Material: Chia seed, flax seed, wheat bran, fennel, Aswagandha, ginger and basil will be used during this study. Plant material will be collected from the local market, Dayalbagh, Agra.

Probiotic Cultures

The probiotic cultures ***Lactobacillus acidophilus, Lactococcus lactis, Lactobacillus bulgaricus and Bifidobacterium*** will be obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal-132001, Haryana, India.

Preparation of Starter Culture

The freeze-dried LAB cultures will be revived in MRS broth by incubating at 40±2°C for 24-48 h. The revived cultures will be re-inoculated in MRS broth and incubated for 12-16 h at 40±1°C. Then 1 ml of the cultures will be transferred into 10 ml of sterile milk and incubated for 12-16 h at 40±1°C. Different combination of the probiotic cultures will be used for the production of various shrikhand samples.

Preparation of Plant Material

Prebiotic, phenolic and antioxidant substances for example Chia Seed, flax seed, wheat bran, fennel, ashwagandha, ginger, basil will be selected as plant sources. These will be cleaned and washed properly with potable water and in last thoroughly washed with distilled water and kept for drying in sunlight for 2 days and kept in a hot air oven at a temperature of 45°C for 6 h. Dried plant material will be powdered and packed in polyethylene bags and stored in an airtight container at refrigeration temperature (4-7°C).

Preparation of Probiotic Shrikhand (using Plant Material Powder)

Toned mile (3.0% fat, 8.5% SNF) will be pasteurized at 72°C for 15 sec and cooled to the temperature of around 4°C. After cooling, the probiotic cultures will be added in the milk @2% inoculum and incubated at 40°C for 15 hours. After incubation, the curd will be drained in muslin cloth for 13 hours to get chakka. Then percentage of sugar and plant material powder added in chakka and kneaded well by using electric blender and stored under refrigeration condition (4°C). Different trials will be done to compare viability of probiotic organism and comparative analysis will be done by combination of probiotic cultures with different plant sources for shrikhand.

Comparison and Storage Studies

The probiotic shrikhand will be packed in polystyrene cups and stored under refrigeration condition (4°C). The packed enriched shrikhand samples conforming to the different treatments will be studied for storage stability during a storage period of 30 days under refrigeration temperature (4°C). The chemical, microbial and sensory analysis of the different samples will be analysed using standard procedure as mentioned by AOAC at regular intervals of 10 days during the period of storage (30 days).

The pH of the shrikhand will be determined using a pH meter.

The moisture content of shrikhand is the loss in mass, expressed as a percentage by mass when the product is heated in a hot air oven.

The total solid will be determined by sodium hydroxide method. In this procedure (IS 12333:1997), NaOH is used for neutralization of developed acidity followed by determination of total solid by evaporation at 100±2°C.

The Fat content will be analysed by Gerber Method (Renner, 1993).

Titratable acidity will be expressed as g of lactic acid /100 g after mixing 10 g of sample with 10 ml of hot distilled water and titrating with 0.1 NaOH using 0.5% phenolphthalein indicator method. The various nitrogenous compounds are covered into ammonium sulphate by boiling with concentrated sulphuric acid. The ammonium sulphate formed is decomposed with alkali (NaOH) and the ammonia liberated is absorbed in excess of the standard solution of acid and then back titrated with standard Alkali.

Organoleptic evaluation

Organoleptic evaluation of different samples will be on the basis of comparison done by 10 semi trained judges at regular intervals of 10 days during 30 days of storage study using nine-point hedonic rating scale to grade different shrikhand with the score ranging from 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much and 9= like extremely. It will judge all parameters by using a scorecard. The numerical score given by the judge for individual attributes will be compute to get mean these means will be subject to statistical analysis.

Viability of Probiotic bacteria in different samples

Viability of probiotic bacteria will be determined by serial dilution method at 10 days intervals after adding plant extract and in storage. One gram of probiotic shrikhand samples will be weighed and serially diluted. The probiotic bacterial population will be enumerated by pour plate technique using MRS media after incubation at 40°C for 48 h. The results will be expressed as log cfu/ml and viability of probiotic microorganism in different samples will be compared.

Microbial quality in different samples

All media will be obtained in dehydrated forms and will be prepared according to the manufacturer's instructions. The enumeration of bacteria will be done using two techniques viz., serial dilution and pour plating using different media. **Plate count Agar** will be used to determine total bacteria count, **Violet Red Biel Agar (VRBA)** will be used to determine the coliform count and **Chloramphenicol Yeast Glucose Agar (CYGA)** will be used to Chloramphenicol yeast glucose agar determine yeast and mould count.

Isolation of *E. coli & Salmonella* from pre-enrichment, enrichment and selective media using **MacConkey broth, E.C. broth** and **EMB Agar** (for *E. coli*) and Buffer **peptone water, Rapports vassiliadis** medium and **Deoxycholate citrate agar.**

Isolation & Charactterization of *Lactobacillus*

De Man, Rogosa and Sharpe (MRS) agar media will be used for selective isolation of *Lactobacillus* colonies from samples. Samples will be streaked across the selected media and incubated at 37°C for 48 hrs. After incubation colonies developing with characteristic growth characters will be isolated and purified and confirmed as LAB by their morphological properties after gram staining, negative catalase test and ability to produce gas in nutrient broth containing 5% glucose (fructose, galactose, lactose, sucrose) at 37°C within 48 hrs. Oxidase test, motility of the *Lactobacillus* sp.

Antimicrobial activity

Antimicrobial activity of all *Lactobacillus* isolates against pathogens will be determined by Agar- well diffusion method as per Ashraf et al., 2009. A 50-100 µl of cell free supernatant of each *Lactobacillus* isolate will be filled in 7mm diameter well in nutrient agar containing test pathogens. The diameter of the inhibition clear zone will be noted after 24 h of incubation.

References

1. VP Jadhav, Jayashri P Hiremath, SD Kalyankar and CD Khedkar, (2019), Studies on Storage stability of probiotic Shrikhand obtained from safflower (Carthamustinctorius)-blended milk, Indian J Dairy Sci 72(2):162-166
2. Rekha Devi, Apoorva Argade, PK Bhardwaj, Vaquil and SS Ahlawat, (2018). Soy milk and fruit based shrikhand: A novel fermented milk product, The Pharma Innovation Journal; 7(3):458-461, 2018

3. Amal Bakr Shori, Fateemeh Aboulfazli, Ahmad Salihin Baba, (2018), Viability of Probiotics in Dairy Products: A Review Focusing on Yogurt, Ice Cream, and Cheese
4. Lalita Oraon, Jana Atanu, Prajapati, Priyanka Suvera, (2017). Application of herbs in functional dairy products – a review, Journal of Dairy, Veterinary & Animal research Volume 5 Issue 3
5. Mahesh Patil1, Manjunatha H2, and Ramachandra B3, (2016). Optimization of carrot enriched probiotic shrikhand, International Journal of Science, Environment ISSN 2278-3687 (O) and Technology, Vol. 5, 2016, 3073-3080, 2016
6. Smriti Gaur* and Anukriti Verma (2017), Evaluation of Probiotic Characteristics of Bacteria Isolated from Fermented Foods, Journal of Pharmacy Research, 11(4), 281-285, 2017
7. Anil Kumar Chauhan, Smita Singh, Ravi Pratap Singh and S P Singh, (2015), Guava-enriched dairy products: a review, Indian J. Dairy Sci. 68(1)
8. Hemalsadrani, Jayantilaldave and Bharat Kumar Rajiv Manuelvyas, (2014), Screening of potential probiotic lactobacillus strains isolated from fermented foods, fruits and of human origin. Vol 7
9. Nidhi Ojha, Dr. Ramesh Chandra, Kamal Rathor, Disha Satwani, Abhishek Kumar and Sherya Srivastava, (2018), Process optimization of herbal shrikhand by incorporating tulsi and turmeric powder. The Pharma Innovation Journal; 7(6): 100-102
10. Suraj Dubey, John David, Gyanendra Gupta and Ganesh Shukla, (2018), Effect of Moringa (Moringaoleifera) powder on quality of Shrikhand. The Pharma Innovation Journal; 7(8): 217-222
11. U.B. Landge, B.K. Pawar and D.M. Choudhari, (2011), Preparation of shrikhand using ashwagandha powder as additive, Asian Journal of Dairy and Food Research. (30): 79-84
12. Reza Mohammadi & Amir Mohammad Mortazavian & Roya Khosrokhavar & Adriano Gomes da Cruz, (2011), Probiotic ice cream: viability of probiotic bacteria and sensory properties. Ann Microbial 61:411-424 DOI 10.1007/s13213-010-0188
13. B. Ray and m. L. Speck, (1978), Plating Procedure for the Enumeration of Coliforms from Dairy Products, American Society for Microbiology.
14. Soomro A.H, M.A. Arain, M. Khaskheli and B. Bhutto, Isolation of Escherichia Coli from Raw Milk and Milk Products, Pakistan Journal of Nutrition 1(3):151-152, 2002

15. Dinker Singh, Jai Singh, Swashankh Kumar and Tarun Verma, (2013). Microbiological evaluation of soy fortified shrikhand by using response surface methodology. International Journal of Applied Biology and Pharmaceutical Technology Volume-5, Issue-1
16. Luciana Albuquerque Caldeira, (2018), Viability of probiotic bacteria in bioyogurt with the addition of honey from Jatai and Africanized bees. Pesq. Agropec. Bras. Vol.53 No.2 Brasilia Feb.
17. Amita Pandey, Shalini Tripathi, (2014), Concept of standardization, extraction and pre-phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry; 2(5):115-119
18. Himanshu Kumar Rai, DC Rai and Saloni, (2018), Study the shelf life of Tulsi (Ocimumtenuiflorum) enriched herbal Shrikhand. The Pharma Innovation Journal; 7(5):611-615
19. C. Prabhurajeshwara, KelmaniChandrakanth b, (2019), Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic Lactobacilli strains isolated from commercial yoghurt. Clinical nutritional experimental 23 97-115
20. Chetan Sharma, 1 Brij Pal Singh, 1 Nishchal Thakur, Antibacterial effects of Lactobacillus isolates of curd and human milk origin against food-borne and human pathogens
21. Mohamed G. Shehata*, Hagar S. Abd-Rabou and Sobhy A. El-Sohaimy, (2019), Plant Extracts in Probiotic Encapsulation: Evaluation of their Effects on Strains Survivability in Juice and Drinkable Yogurt During Storage and an in –vitro Gastrointestinal Model. Shehata et al. J Pure Applied Microbial, 13(1), 609-617
22. Antonia Terpou, Aikaterini Papadaki, Iliada K. Lappa, Vasiliki Kachrimanidou, Loulouda A. Bosnea, and Nikolaos Kopsahelis, (2019), Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. Nutrients, 11(7), 1591
23. Belay Telahun, 1 AntenehTesfaye, (2018) Isolation and Molecular Identification of Lactic Acid Bacteria Using 16s RNA Genes from Fermented Teff (Eragrostistef (Zucc.)) Dough. International Journal of Food Science Volume, Article ID 8510620
24. Omar B Ahmed, Atif H Asghar, Ibrahim HA Abd El-Rahim and Hegazy AI, Detection of Salmonella in Food Samples by Culture and Polymerase Chain Reaction Methods

25. Waldemargustaw, (2011), The Influence of Selected Prebiotics on the Growth of Lactic Acid Bacteria for Bio-Yoghurt Production, Sci Pol Technol Aliment, 10(4):455-66
26. Younis K, Ahmad S and Jahan K., (2015), Health Benefits and Application of Prebiotics in Foods. Younis, et al., J Food Process Technology, 6:4 DOI: 10.4172/2157-7110.1000433
27. J.K. Momin, J.B. Prajapati, (2019), Effect of selected medicinal herbs on viability and acid production of lactic dairy starters, Emer Life Science Res, 5(2):35-42
28. KamrunNaharIslam, Touaha Akbar, FahmidaAkther and NazneenNaher Islam, (2016), Characterization and Confirmation of Lactobacillus spp. From Selective Regional Yoghurts for Probiotic and Interference with Pathogenic Bacterial Growth, Asian Journal of Biological Sciences ISSN 1996-3351
29. Shilpa Sharma, Ravinder Kaushik, (2016), Effect of herb extract on growth of probiotic culture.